INTRODUCTION

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism associated with a number of human neurodegenerative disorders, diabetes, cardiovascular diseases, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Free radicals are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones (adrenalin and noradrenalin). Accumulated evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants. Africa continent has enormous biodiversity resources, but plagued with several diseases, including those associated with ROS overproduction as one of the etiological factor. Therefore, therapy using free-radical scavengers (antioxidants) has potential to prevent, delay or ameliorate many of these disorders (Delanty and Dichter, 2000). Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis (Surh and Fergusson, 2003; Park and Pezzuto, 2002; Wattenberg, 1996; Greenwald, 2002; IARC, 1996; Fujiki, 1999; Tsao et al., 2004; Kinghorn et al., 2004; Mehta and Pezzuto, 2002). Similar evidence also exist to demonstrate the chemopreventive capacities of ethnobotanicals and components of vegetable diets with free-radical scavenging potential on ulcers (Borreli and Izzo, 2000), diabetes (Sahu and Kuttan, 2002), memory and cognitive function (Howes and Houghton, 2003), Alzheimer’s disease (Howes et al., 2003; Perry et al., 1998), age-related neurological dysfunction (Youdim and Joseph, 2001; Delanty and Dichter, 2000), cardiovascular and renal disorders (Anderson et al., 1999; Miller, 1998) and several other human ailments (Scartezzini and Speroni, 2000; Borek, 2001; Craig, 1999; Galvano et al., 2001; Lampo, 2003; Surh, 1999).

Tapinanthus globiferus, popularly called ‘afomo’ in South Western Nigeria is a parasitic plant known to grow on different trees. There has been no report about its free phenolic content and flavonoid compounds. Furthermore, little is known about the antioxidant potential of this plant. Although T. globiferus is being used in folkloric medicine in the management of free radical related disorders, there is little or no information about its phytochemical constituents and antioxidant activities which may be involved in its antihypertensive ability. This study is designed to determine the phenolic acid and flavonoid contents, as well as antioxidant activities of T. globiferus.

MATERIALS AND METHODOLOGY

Chemicals

All chemicals used including solvents, were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin Ciocalteu’s phenol reagent, malonaldehyde bis-(dimethyl acetal) (MDA), Thiobarbituric acid (TBA), sodium dodecyl sulfate, ascorbic acid, 2,7’-dichlorofluorescein diacetate (DCFH-DA), Tris-HCl ethylene glycol tetraacetic acid (EGTA), quercetin, rutin, chlorogenic acid and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), ferrous sulfate, mannitol, sucrose were obtained from Vetec (Rio de Janeiro, RJ, Brazil).

Plants Extract and Extraction procedure

Leaves of Tapinanthus globiferus was obtained from Ogbomoso, Nigeria in 2011 and was identified by Dr. Ogunkunle of the Botany Unit, Department of Pure and Applied Biology and were confirmed.
with a plant name index. The leaves were dried under room temperature. The dried leaves were grinded into a powdery form and the powder of T. globiferus (100 g) was macerated at room temperature with ethanol (70%) and extracted for 72 hours at the Biochemical Toxicological Unit, Department of Biological sciences, Federal University of Santa Maria, Santa Maria RS, Brazil. On the third day, the combined ethanolic extract was filtered and the solvent was fully evaporated under reduced pressure to give a green solid. The ethanolic extract was then diluted in distilled water in order to prepare different concentrations (10, 50, 100, and 250 μg/mL).

**DPPH radical scavenging activity**

The free radical scavenging activity of T. globiferus extract was measured with the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Howes et al., 2003) in terms of hydrogen-donating or radicals scavenging activity according to the method described by Bandonie and Murkovic (2002). A solution of DPPH (0.3 mM) in ethanol was prepared, and 100 μL of this solution was added to 20 μL aqueous extract at different concentrations (10, 50, 100 and 250 μg/mL).

![AH = antioxidatnt compound DPPH = 1,1-diphenyl-2-picrylhydrazyl](Image)

**Fig 1: The In Vitro chemical representation of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay.**

Distilled water and ascorbic acid at the same concentrations used for aqueous extract were used as negative and positive controls respectively. After 30 minutes, absorbances were measured at 548 nm in ELISA plate reader (TP-Reader, China).

The free radical scavenging capacity of the extract is calculated with the equation:

\[
\% \text{ inhibition} = \frac{\text{Control Absorbance} - \text{Test Absorbance}}{\text{Control Absorbance}} \times 100
\]

**Total phenolic content**

The total phenolic content of T. globiferus was estimated according to the method of Makkar et al. [21] with minor modifications. Samples of the extract (10 - 250 μg/mL) were added to a test tube and the volume was adjusted to 1.4 mL with distilled water. Then, 0.2 mL of Folin-Ciocalteau reagent (diluted 1:1 with water) and 0.4 mL of sodium carbonate solution (7.5%) were added sequentially to the test tube. The tubes were then incubated for 40 min at 45°C and the absorbance was measured at 725 nm in a spectrophotometer (SP-2000UV). The standard curve was prepared using 0, 1, 2.5, 5, 10 and 15 μg/mL solutions of gallic acid (0.1 mg/mL). Total phenol value was calculated and expressed as microgram gallic acid equivalent (μg GAE)/g of dry extract.

**Iron-chelating activity**

The method described by Benzie and Strain (1996) was adopted for the assay. The principle is based on the formation of O-Phenanthroline-Fe²⁺ complex and its disruption in the presence of chelating agents. The reaction containing 40ul of ferric chloride (3.12mM), 740μl of Tris-HCl (0.1M) and different volumes of different concentrations of extract was made up to 2ml with distilled water and was incubated for 5 minutes. The mixture was used to zero the wavelength of the spectrophotometer. Twenty five microliter of ortho-phenanthroline was thereafter added to the mixture in the cuvette and absorbance taken at 510nm. The Fe²⁺ chelating capacity was calculated thus:

\[
\text{Fe}^{2+} \text{ chelating activity (}) = \frac{(Ac - As)/Ac} \times 100
\]

**Animals**

Male Wistar rats weighing 270-320 g and with age from 2.5 to 3.5 months from breeding colony (Animal House-holding, USFM, Brazil) were used for the study. They were kept in cages with free access to foods and water in a room with controlled temperature (22 ± 3°C) and in 12 h light/dark cycle. The protocol has been approved by the guidelines of the Brazilian association for laboratory animal science (CONCEA).

**Quantification of phenolics and flavonoids compounds by HPLC-DAD**

Free phenolic and flavonoid contents were determined using High performance liquid chromatography (HPLC-DAD). This was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominance Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SPL. Briefly, reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm x 250 mm) packed with 5μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 65 min, respectively, following the method described by Laghari et al. (2011) with slight modifications. The extracts of T. globiferus were analyzed, at a concentration of 5 mg/mL. The presence of six phenolics compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 ml/min, injection volume 40 μl and the wavelength were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031 – 0.250 mg/ml for kaempferol, quercetin and rutin; and 0.006 – 0.250 mg/ml for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). Calibration curve for gallic acid: Y = 11611x + 1468.8 (r = 0.9999); chlorogenic acid: Y = 14762x + 2575.7 (r = 0.9997); caffeic acid: Y = 11526x + 1293.1 (r = 0.9995); rutin: Y = 13035x – 1045.9 (r = 0.9998); quercetin: Y = 15105x – 1192.3 (r =0.9998) and kaempferol: Y = 15232x – 1303.9 (r = 0.9999). All chromatography operations were carried out at ambient temperature and in triplicate.

**Isolation of rat liver mitochondrial**

Rat liver mitochondrial was isolated as previously described by Puntel et al. [2010] with some modifications. The livers were rapidly removed (within 1 min) and immersed in ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM K²EGTA and 10 mM K²HEPES, pH 7.2. The tissue was minced using surgical scissors and then extensively washed. The tissue was then homogenized in a power-driven, tight-fitting Potter-Elvehjem homogenizer with Teflon pestle. The resulting suspension was centrifuged, for 7 min at 2000 g in Hitachi CR 21E centrifuge. After centrifugation, the supernatant was centrifuged for 10 min at 12000g. The pellet was resuspended in “isolation buffer II” containing 225 mM mannitol, 75 mM sucrose, 1 mM K²EGTA, and 10 mM K²HEPES, pH 7.2, and recentrifuged at 12,000g for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in respiration buffer containing 100 mM sucrose, 65 mM KCl, 10 mM K²EGTA and 50 mM EGTA, pH 7.2, to a protein concentration of 0.6 mg/mL.
Generation and measurement of reactive oxygen species (ROS)

ROS production in isolated mitochondria was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescence probe. Mitochondrial suspensions (0.25 mg/mL) in respiration buffer containing 100 mM sucrose, 65 mM KCl, 10 mM K+–HEPES and 50 μM EGTA, pH 7.2 were incubated with different concentrations of the extract (10–250 μg/mL) in the presence or absence of 12μM of H2O2. Then, 3.33 μM of DCFH-DA; then, 10, 50, 100, 250 μg/mL of ethanolic extract [Ketson and Brandt, 1965; Kinghorn et al., 2004; Lampe, 2003] were added to the solution. The formation of the oxidized fluorescent derivative (DCF) was monitored using a spectrofluorimeter (Shimadzu RF-5301) with excitation and emission wavelengths of 488 and 525 nm respectively and with slt widths of 1.5 nm.

Statistical analysis

Values were expressed as mean ± SEM (standard error of mean). Statistical analyses were performed by one way ANOVA, followed by Duncan’s multiple range tests. The results were considered statistically significant for p < 0.05.

RESULTS

Iron- chelating capacity

Iron binding of ethanolic extract of T. globiferus at different concentrations was determined and the values were summarized in Table 1. The iron-chelating activity of T. globiferus was dose-dependent with IC50 at 247.120±0.914.

Table 1: Effects of ethanolic extract of the leaves of T. globiferus on iron chelation.

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>%Fe²⁺ chelation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>21.04±0.32</td>
</tr>
<tr>
<td>10</td>
<td>24.56±0.30</td>
</tr>
<tr>
<td>20</td>
<td>26.47±0.44</td>
</tr>
<tr>
<td>50</td>
<td>29.68±0.61</td>
</tr>
<tr>
<td>100</td>
<td>37.76±0.96</td>
</tr>
<tr>
<td>250</td>
<td>53.62±0.80</td>
</tr>
<tr>
<td>IC50 (μg/mL)</td>
<td>247.12±0.91</td>
</tr>
</tbody>
</table>

DPPH Assay

DPPH color was quenched by vitamin C (Table 1 or Figure x) and in a concentration dependent manner by alcholic extract from T. globiferus and a maximal inhibitory effect of about 90% was obtained with the highest concentration tested (250 g/ml).

Table 2: Percentage free radical inhibition by the ethanolic extract of Tapinanthus globiferus (DPPH).

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>10μg/ml</th>
<th>50μg/ml</th>
<th>100μg/ml</th>
<th>250μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic</td>
<td>75.35±3.1</td>
<td>78.43±2.4</td>
<td>80.49±2.2</td>
<td>80.76±2.4</td>
</tr>
<tr>
<td>Acid</td>
<td>8%</td>
<td>3%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>T. globiferus</td>
<td>22.95±1.8</td>
<td>60.89±2.2</td>
<td>78.23±4.7</td>
<td>88.75±1.2</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total phenolics

The amount of total phenolic of ethanolic extract of leaves of T. globiferus estimated by Folin-Ciocalteau method was 2.77±1.24 mgGAE/g of dry extract.

Determination of phenolic and flavonoid contents in T. globiferus

HPLC fingerprinting of extract revealed the presence of the phenolic acids such as gallic acid (tR = 12.19 min), chlorogenic acid (tR = 21.58 min), and caffeic acid (tR = 24.97 min), while the flavonoid contents included rutin (tR = 38.03 min) and quercetin (tR = 45.11 min) (Fig. 1 and Table 3). The highest of the estimated phenolic acids in the ethanolic extract of T. globiferus was chlorogenic acid (5.81±0.05) while the least was caffeic acid (1.77±0.01). The predominant of the estimated flavonoid contents is rutin (9.12±0.09).

Table 3 - Free phenolic and flavonoid compositions of Tapinanthus globiferus extracts by HPLC/DAD.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>TC Mg/g</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>2.09±0.13a</td>
<td>0.20</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>5.81±0.05b</td>
<td>0.58</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.77±0.01a</td>
<td>0.18</td>
</tr>
<tr>
<td>Rutin</td>
<td>8.12±0.09c</td>
<td>0.81</td>
</tr>
<tr>
<td>Quercetin</td>
<td>7.03±0.02c</td>
<td>0.70</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters in the column differ by Tukey test at p < 0.05.

Figure 4 – Representative high performance liquid chromatography profile of Tapinanthus globiferus extract. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4) and quercetin (peak 5).

A & B= +ve & -ve controls respectively; C, D, E & F = ROS production at concentrations of 10μg/ml, 50μg/ml, 100μg/ml & 250μg/ml respectively.
Antioxidative activity of *T. globiferus* was estimated by assessing its iron chelating capacity. This method is based on the fact that 1,10-phenanthroline will chelate iron II ion to form a complex. However, any other chelator if present in the medium will interfere with the formation of iron II complex by 1, 10-phenanthroline. Measurement of the rate of reduction of the colour allows estimation of the iron chelating ability. The presence of *T. globiferus* extract in the reaction mixture interferes with this by chelating the iron II ion thereby forming complex with the iron II ion. Even at concentration as low as 5µg/ml, ethanolic extract of the plant demonstrated high capacity for iron chelation. This shows that *T. globiferus* possesses iron chelating capacity which increases with increasing concentrations of *T. globiferus*. Although iron is essential to life because of its requirements in various physiological and biochemical processes such as oxygen transport, respiration and its involvement in enzymatic activities, however, it has been implicated in the oxidative damages in lipids, proteins and other cellular components leading to occurrence of diseases such as cardiovascular and neurodegenerative diseases. Hence its control is important for normal functioning of the body. There have been reports that chelating agents which form 0-bonds with a metal ion are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidized form of the metal ion (Gordon, 1990). This data showed that *T. globiferus* may be a good antioxidant due to its demonstration of high iron chelating capacity.

The ability of Ethanolic extract of *T. globiferus* to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). This was compared with a standard (ascorbic acid). Ethanolic extract of *T. globiferus* demonstrated high capacity for scavenging free radicals as shown by the data by reducing the stable radical DPPH to the yellow coloured diphenylpicryl hydrazine and this capacity increases with increasing concentration. Furthermore, comparison with ascorbic acid (standard), showed *T. globiferus* to compete favourably. This demonstrates its high anti-oxidative activity with its IC50 at 38.59µg/ml. The scavenging ability of the *T. globiferus* extract may be due to its bioconstituents such as phenolic acids and flavonoid. However, presence of other biomolecules in its extract may contribute to its free radical scavenging activity.

High performance liquid chromatographic determination (HPLC) showed presence of phenolic acids such as gallic acid, chlorogenic acid and caffeic acid in the ethanolic extract of *T. globiferus* while the flavonoid contents included rutin and quercetin. The highest of the estimated phenolic acids in the ethanolic extract of *T. globiferus* was chlorogenic acid (5.81±0.05) while the least was caffeic acid (1.77±0.01). One of the phenolic constituents of *T. globiferus* is gallic acid. Gallic acid is commonly used in the pharmaceutical industry because many in vivo and in vitro studies in humans, animals, and cell culture have provided evidence for the following actions of gallic acid: (1) it shows cytotoxicity against cancer cells, without harming healthy cells (Ekiri et al., 2006); (2) it can be used to treat albuminuria and diabetes (Chiu-Lan et al., 2007); (3) it seems to have antifungal and antiviral properties (Misao et al., 2007); (4) used as an antioxidant and helps to protect human cells against oxidative damage (Jittawan and Sirithon, 2008; Aref et al., 2007); (5) it can be used as a remote astringent in cases of internal hemorrhage (Hurrell et al., 1999); (6) used to treat psoriasis and external hemorrhoids containing gallic acid (Cook et al., 1995).

Caffeic acid has been shown to inhibit carcinogenesis, although other experiments show possible carcinogenic effects. It is also known as an antioxidant in vitro and also in vivo (Otho et al., 2001). Caffeic acid also shows immunomodulatory and antiinflammatory activities. Caffeic acid and its derivative, Caffeic acid phenethyl ester (CAPE) have shown tumor-shrinking properties. The subcutaneous and oral administrations of caffeic acid and CAPE significantly reduced liver metastasis (Chung et al., 2004). A study using the caffeic acid phenethyl ester (CAPE) showed a positive effect on reducing carcinogenic incidence. It is known to have antimicrobial, anticarcinogenic, anti-inflammatory, and immunomodulatory properties (Natarajan et al., 1996). Another study also showed that CAPE suppresses acute immune and inflammatory responses and holds promise for therapeutic uses to reduce inflammation (Orban et al., 2000).

The predominant of the estimated flavonoid contents is rutin (81.22±0.09). The relationship between the chemical structure of flavonoids and their radical-scavenging activities was analyzed by Bors, Heller, Michael, and Saran (1990). Quercetin has a catechol structure in ring B, as well as a 2, 3-double bond in conjunction with a 4-carbonyl group in ring C, allowing for delocalization of the phenoxyl radical electron to the flavonoid nucleus. The combined presence of a 3-hydroxy group with a 2, 3-double bond additionally increases the resonance stabilization for electron delocalization; hence it has a higher antioxidant value. This chemical structure and its antioxidative relevance might have contributed to the overall antioxidant activity of *T. globiferus*. Rutin is a glycoside of the flavonoid quercetin. As such, the chemical structures of both are very similar, with the difference existing in the Hydroxyl functional group. Both quercetin and rutin are used in many countries as medications for blood vessel protection, and are ingredients of numerous multivitamin preparations and herbal remedies. In humans, it attaches to the iron ion Fe++, preventing it from binding to hydrogen peroxide, which would otherwise create a highly-reactive free radical that may damage cells. It is also an antioxidant. Furthermore, it has been shown to inhibit in vitro the vascular endothelial growth factor (Hattori et al., 2008) in subtoxic concentrations, so acts as an inhibitor of angiogenesis. This finding can be potentially relevant for the control of some cancers. Also there has been report that rutin inhibits ovariectomy-induced trabecular bone loss in rats, both by slowing down resorption and increasing osteoblastic activity (Horcajada et al., 2000).
Under normal physiological conditions there is equilibrium between reactive oxygen species (ROS) generated and antioxidants present. The ROS generated is kept in check by antioxidant defense cascade consisting of enzymatic and non-enzymatic components. One specific ROS, hydrogen peroxide (H₂O₂), which is generated by mitochondrial respiration through a specialized enzyme, is a potent inducer of oxidative damage and mediators of ageing. Here, oxidative damage was stimulated by H₂O₂. DCFH-DA was first described as a probe to evaluate H₂O₂ (Keston and Brandt, 1965); subsequently, it has been suggested that increases in DCF fluorescence actually reflect the overall cellular oxidative stress (Wang and Joseph, 1999), since other forms of free radicals such as peroxyl radical, peroxinitrite, nitric oxide can oxidize DCFH. Our results indicated that H₂O₂ caused a significant increase in ROS production and that ethanolic extract of T. globiferus was able to prevent significantly ROS production stimulated by H₂O₂ in a concentration-dependent manner. This effect may be attributed to the activities of quercetin and rutin found in plant extract. In fact, recently, our laboratory have reported that quercetin and its glycoside analog, rutin, prevents against methylmercury–induced osteopenia in rats. Bone Miner Res. 15(11):2251-8.

In conclusion, ethanolic extract of T. globiferus could be a good raw material for the development of drugs useful for the treatment of chronic inflammatory diseases.

REFERENCES


32. Natarajan K, Singh S, Burke TR, Grunberger D, Aggarwal BB (1996). Caffeic acid phenethyl ester is a potent and specific